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TITLE: "Role of Ca⁺⁺ Influx via Epidermal TRP Ion Channels"

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| 14. ABSTRACT To benefit military veterans with amputations who suffer skin problems on their amputation stumps, this proposal describes mechanistic studies to pave the way for novel methods of improving skin barrier function at the residual limb-prosthetic interface. Signaling systems in skin will be modulated to increase barrier function, attenuate irritant dermatitis, and characterize the underlying signaling mechanisms so that they can become better targets for treatment. Progress in year 2 of the funding period is described in this Annual Progress Report. We maintained all the necessary regulatory approvals from the Durham VA, Duke University IRB and the DoD to conduct the human experimentation. We set up experiments in primary skin cells for mechanical stress, which we found disrupts skin barrier function. We also found that activation of ion channel TRPV4 can re-normalize barrier function of the skin that has been disrupted by mechanical stress. We also found this particular pattern for keratinocytes' regulatory volume decrease, as a surrogate of their capability to moisturize. | | | | | |
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Introduction

The objective of our proposal is to benefit military veterans with amputations who suffer skin problems on their amputation stumps. To fulfill the objective, this proposal describes mechanistic studies to pave the way for novel methods of improving skin barrier function at the residual limb-prosthetic interface.

Rather than attempting to enhance the functions of barrier-enhancing proteins, filaggrin (structural protein in upper epidermal layers) and aquaporins (water channel) by individual targeting, we thought it an attractive (and testable) idea, whether targeting of any of the epidermal transient receptor potential ion (TRP) ion channels could lead to **improved** barrier function and thus better moisturization, thus less irritation, injury and pain. This would be accomplished by affecting both, filaggrin and aquaporin. In keeping with our reasoning, recent insights on activation of TRPA1 and TRPV4 in the skin are supportive of our concept. However, an obstacle toward improved insight is that mammalian skin was not studied in response to mechanical stress in order to wear down the barrier, so that its more rational repair, by targeting specific signaling mechanisms of the epidermis, can be assessed.

Hypothesis

Therefore, the central hypothesis of our proposal is that Ca^{++} influx into epidermal keratinocytes – mediated via TRP ion channels that we intend to activate specifically - controls moisturization and barrier function of the skin by critically influencing filaggrin- and aquaporin function in a cell-autonomous manner, and that activation of these TRP channels can be helpful for repairing a compromised barrier which is caused by injurious mechanical stress.

Specific Aims

- (1) to assess skin barrier function and moisturization parameters in response to modulation of TRPV1, 3 and 4 and TRPA1 in normal human skin as it is subjected to mechanical stress.
- (2) to assess skin barrier function and moisturization in epidermal cultures derived from military veterans with amputations and stump skin irritation, and to determine the epidermis' response to activation of TRP channels.

Keywords

Amputation stump, irritant dermatitis, epidermis, skin barrier function, TRP channel, aquaporin (AQP) channel, UVB, UVA

Accomplishments

Approval for human experimentation

We continue to maintain approval by the Durham VA and Duke University IRBs.

Human artificial skin

Again, we obtained human EpiDermF (Mattek; IRB exempt) and grew the artificial skin in culture. We adapted it to the revised mechanical stress apparatus (Flexcell Devices), obtaining improved reliability when subjecting the artificial human skin to mechanical stress, improved consistency and increased strain levels (Fig. 1).

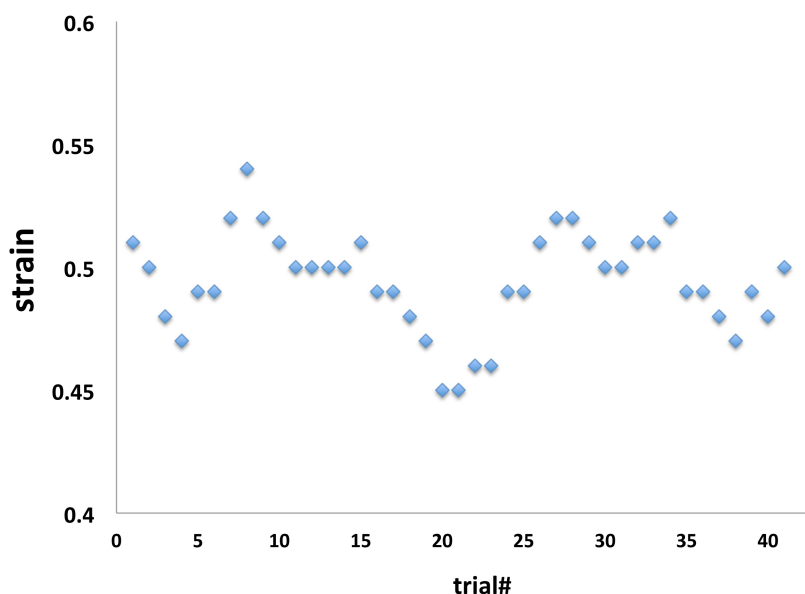


Fig. 1: Targeted strain level of 0.5 in 40 trials. Note variation when targeting strain level of 0.5 in cyclical mechanical compression device (bioreactor).

Measurement of skin capacitance in culture

Using our NOVA capacitance meter with flexible probe, DPM9003BT, we established skin capacitance from human artificial skin in culture. We were able to successfully build on progress as outlined in the previous progress report. Increase of capacitance in response to topical glycerol was consistent, along the lines as reported in the previous report.

Applying cyclic mechanical strain for up to 0.5 strain, we were able to maintain the mechanical stress for several days. In general, metrics of damage tended to be maximal at the end of the test period. We ran experiments as long as 10 days, with cyclical strain, 1h on, 1h off, for 12h, with 12h rest.

We next stimulated TRPV4 channels by using activation with GSK101. As a result, capacitance went up dose-dependently, was abolished using GSK205 TRPV4 selective inhibitor (Fig. 2).

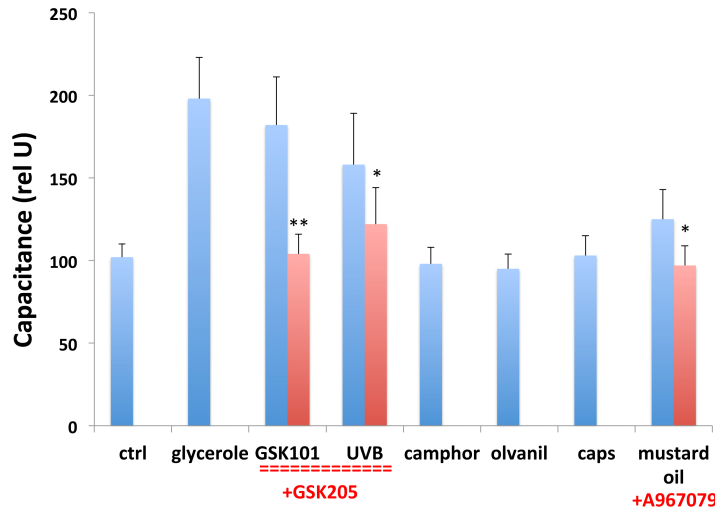


Fig. 2: Human artificial skin capacitance in response to TRP ion channel activation. Capacitance was measured with our skin capacitance meter with flexible probe, DPM9003BT. Treatment with 5% glycerol consistently almost doubled the capacitance, as reported in previous progress. Activation of TRPV4 with GSK101 (50nM) and UVB (150mJ/cm², 5x15 seconds with 60 sec interval) led to capacitance increase that could be attenuated with TRPV4 selective inhibitor, GSK205 (10μM). Stimulation with camphor (TRPV3), olvanil and capsaicin (TRPV1) did not change capacitance, whereas stimulation with mustard oil (TRPA1; 100 μM) did, which was attenuated by TRPA1-selective inhibitor, A967079 (5μM). Matek EpidermF was used, n≥8 experimental repeats per experimental group, with 10 technical repeats per experimental repeat. * p<0.05, ** p<0.01, Student's t-test

Increase in capacitance, using UVB stimulation was not as high, but could be reliably accomplished, with slight increase in exposure, but not longer than for 20 seconds. Again, this capacitance could be blocked by GSK205. – More experiments are being conducted at the time of writing.

When activating TRPV3 with camphor, and TRPV1 with capsaicin and olvanil, there was no significant increase in capacitance. In view of this finding, blockers for these channels were not used.

In response to TRPA1 activation, using mustard oil, we were able to observe approximately 20% of the TRPV4-mediated effect, which could be blocked with specific TRPA1 inhibitor. Attempts to activate TRPA1 with UVA were met with tendential increase, but large variation could be observed.

Compounds were applied in 2% DMSO-vaseline. This vehicle proved inert in affecting skin capacitance.

In 2D primary dissociated human KC culture, derived from EpidermF, we observed that regulatory volume decrease, in response to 240mosmol/L, was impaired after cells were cultured after mechanically stressing the artificial skin (strain 0.5, sinusoidal stress 0.2Hz, 1h stim/1h rest, 12h stimulation, 12h rest) for 2 days. Activation of TRPV4 with GSK101 could re-normalize the compromised RVD. Activation of TRPV3, TRPV1 and TRPA1 did not have an effect (Fig. 3).

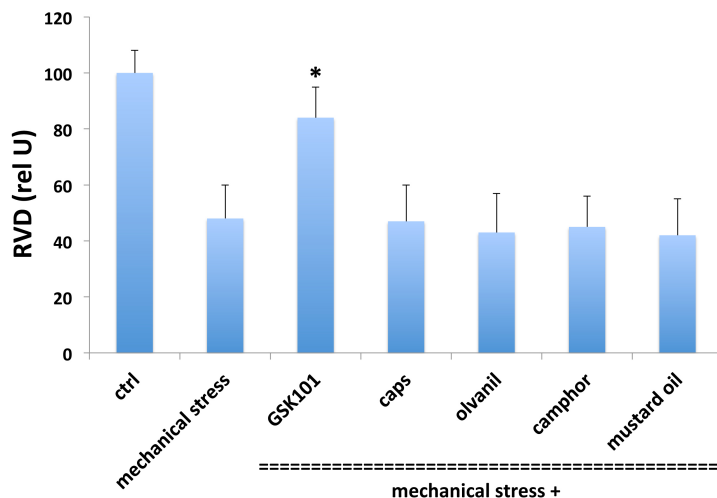


Fig. 3: Human keratinocyte regulatory volume decrease is impaired by chronic mechanical stress, can be restored in response to selective stimulation of TRPV4 (GSK101 50nM; * $p < 0.05$), not TRPV1, TRPV3 and TRPA1. N=4 experimental replicates, ≥ 50 keratinocytes per replicate

Stress-testing of human artificial skin

EpiDermF were grown in culture. We applied mechanical stress at strains of 0.3, 0.4 and 0.5, using our improved Flexcell Bioreactor Compression system. No signs of mechanical/ gross structural damage were registered. However, we believe that strain levels of 0.5 can be considered injurious, especially with chronic sinusoidal application. We applied sinusoidal compression at 1/5 sec (0.2Hz) for 1h, followed by an hour rest, for 12h, then 12h rest. We observed that barrier function became impaired, when applying this mechanical stress protocol. This was suggested by decreased capacitance, which we measured every day, detecting a gradual decrease of barrier function, peaking at d10, the longest time-period that we have applied mechanical stress so far (Fig. 4).

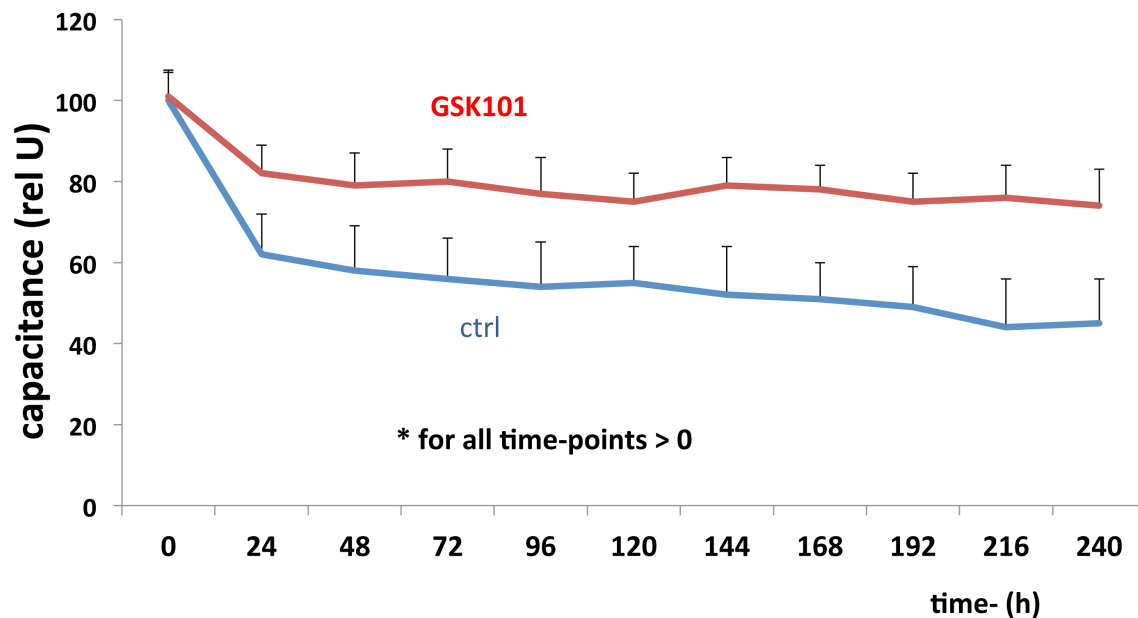


Fig. 4: Decline of human artificial skin capacitance in response to chronic mechanical stress (see narrative). Note rescue of decline of capacitance, caused by mechanical stress, when chemically activating TRPV4 ion channels. N=6 experimental replicates; * denotes $p < 0.05$, t-test.

Given our results with stimulation of TRPV4 channels, we demonstrated rescue using chemical stimulation of TRPV4 with GSK101 (50nM for 5 min during the rest interval, 3x/d; Fig. 4). Using UVB, some attempts were quite successful to

re-normalize barrier function, other experiments were characterized by higher levels of variation. We are currently addressing these experimental difficulties.

Transcript levels in mechanically-stressed human artificial skin

EpiDermF was subjected to strains of 0.3, and 0.5 for 1 day, using our cyclical protocol, as described above. From these skin samples (n=7 mechanically stressed, n=6 baseline), we extracted RNA and subjected them to reverse transcriptase, followed by quantitative PCR.

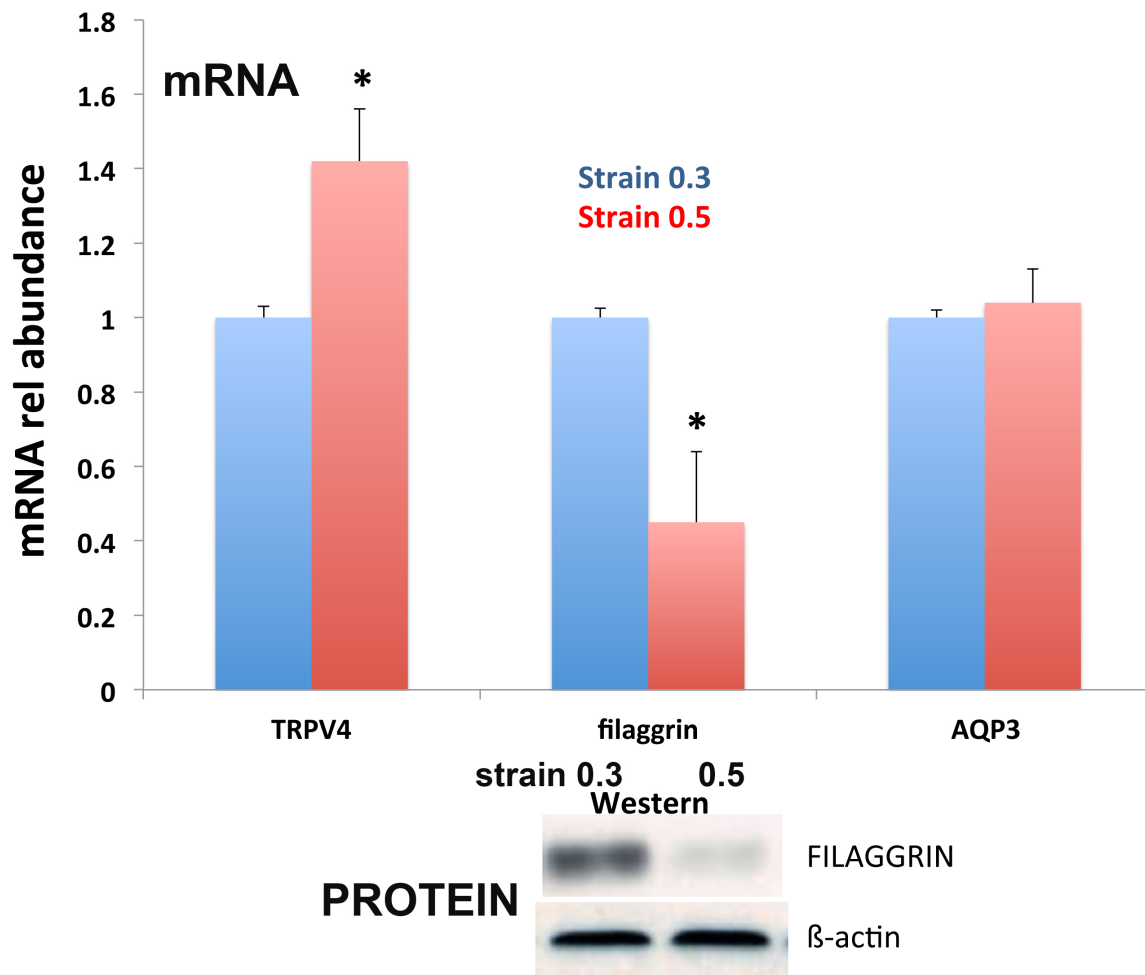


Fig. 5: Gene expression in response to mechanical stress. Note moderate increase of TRPV4 expression, significant decline of filaggrin, both at the mRNA and protein level, and no change in AQP3 expression level.

We detected all TRP ion channels under study (TRPV1, TRPV3, TRPV4, TRPA1), filaggrin and AQP3. As observed in y1, there were no gene-expression

changes at a strain rate of 0.3. At 0.5, TRPV4 transcripts were moderately upregulated, AQP3 was unchanged, and filaggrin transcripts were down-regulated, a finding mirrored by filaggrin protein levels, assessed by WB (Fig. 4). We then attempted rescue of diminished filaggrin expression, a telling surrogate of skin barrier dysfunction, by activation of TRPV4 with GSK101. Initial results were encouraging, and we are currently working on establishing the most effective protocol at stimulating TRPV4 in order to obtain the most effective barrier repair.

Impact

We view the finding that selective activation of TRPV4 channels in skin can increase barrier function and re-normalize impaired regulatory volume decrease as a reportable outcome that has general as well as human health-related relevance.

Changes/Problems

The mechanical stress apparatus appears to work more reliably. We aim for accomplishing a reliable and consistent strain of 0.6 with it.

UVA activation of TRPA1 was not very consistent, UVB activation of TRPV4 showed some variation, indicating the need for experimental refinement. TRPV4 chemical activation using GSK101 in order to improve barrier dysfunction can also benefit from more refinement, just as was done in a recent study with TRPV4+ chondrocytes, where one particular protocol led to the most effective switch-on of TRPV4 driving anabolic function of chondrocytes, whereas slightly different stimulation protocols using GSK101 were vastly less effective.

Defining damage to the skin barrier elicited by mechanical stress by using Lucifer yellow has not been very reliable, up to this point. We are trying to address this point, harboring some confidence to be able to realize this because it is a well-established method.

Human Experimentation

Challenges in recruiting human subjects remain.

Our efforts to facilitate recruitment did not gain the expected traction. As a result, we intend to improve the efficiency of reaching out to affected veterans by reaching out to a higher number of possible candidates.

Products

As mentioned under “Impact”, we view the finding that selective activation of TRPV4 channels in human skin can increase barrier function, apparently also valid for diminished barrier function as a result of mechanical stress, as a reportable outcome that has general as well as human health-related relevance.

Participants

Liedtke, Wolfgang MD, PhD; Associate Professor of Neurology – PI

Yeo, Michele PhD; Senior Research Associate

Kanju, Patrick PhD; Senior Research Associate

Jin, Yingai; Research Technician

Dr Yong Chen, listed as Participant in the previous progress report, obtained his own NIH fellowship grant, as part of an institutional K12 training grant to prepare for his faculty-independence, and had to pursue the goals of his training at 100% effort, as mandated by the NIH. His share of the projected work was taken over by 2 senior research associates within the Liedtke-Lab (partial effort), who were conducting the cellular work and mechanical stress testing (Dr Yeo), and the testing of functional ion channel activation (Dr Kanju).

Special Reporting Requirements

Quad Chart attached.

Appendices

N/A

Role of Ca^{++} influx via epidermal TRP ion channels

W81XWH-13-1-0299 – Peer-reviewed orthopaedic research program: idea development award
OR120114



PI: Dr. Wolfgang Liedtke

Org: Duke University, Durham NC

Award Amount: US\$ 775,575.00 (total cost over 3y)

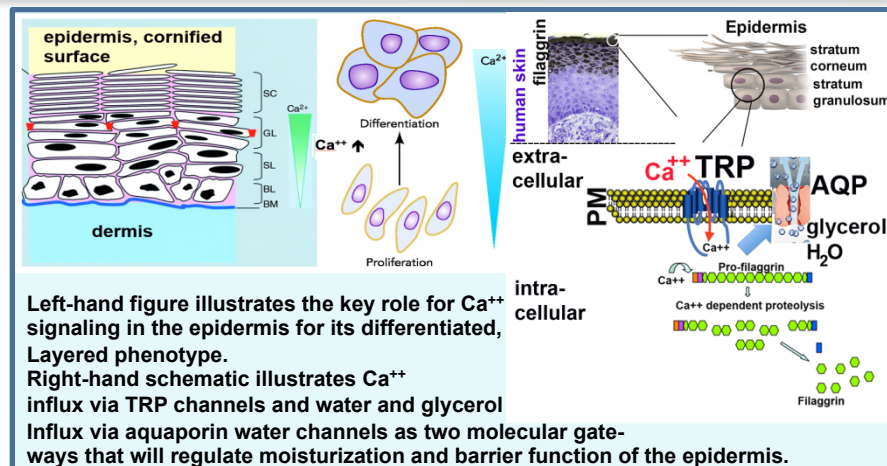
Study Aim(s)

(1) to assess skin barrier function and moisturization parameters in response to modulation of TRPV1, 3 and 4 and TRPA1 in normal human skin as it is subjected to mechanical stress.

(2) to assess skin barrier function and moisturization in epidermal cultures derived from military veterans with amputations and skin irritation of the prosthesis interface of the residual limb, and to determine the epidermis' response to activation of TRP channels.

Approach

We will specifically activate TRP ion channels in human skin keratinocytes and assess whether this has a beneficial effect on skin barrier formation and moisturization state. Human skin organotypic preparations will be used as a generic model (Aim 1), in addition skin biopsies and cultured keratinocytes from military veterans with skin irritation and dysfunctional barrier and impaired moisturization at the prosthesis interface of the residual limb (Aim 2).



Accomplishments: (1) demonstrated barrier dysfunction caused by mechanical stress of human artificial skin; (2) demonstrated that activation of TRPV4, and to lesser degree TRPA1 channels, can attenuate mechanical-stress mediated skin barrier dysfunction; (3) maintained human experimentation approvals.

Timeline and Cost

| Activities | CY | 14 | 15 | 16 |
|------------------------------------------------------------------------------------------------|----|-------|-------|-------|
| Characterize mechanical stress response in cultured human skin | | | | |
| Modulate mechanical stress response in cultured human skin | | | | |
| Assess skin barrier function in epidermal cultures from mil veterans | | | | |
| To define the response of cultured skin cells from military veterans to TRP channel activation | | | | |
| Estimated Budget (\$K) | | \$166 | \$166 | \$166 |

Goals/Milestones

CY15 Goals

- ☐ Characterize and modulate mechanical stress response in cultured human skin. – **Generated significant progress.**
- ☐ Assess and modulate skin barrier function in epidermal samples and cultures from military veterans with irritant dermatitis of their residual limb and healthy controls. – **Maintained regulatory approval for human experimentation, engaged in measures to improve recruitment.**

CY16 Goal - as CY15 Goals

Comments/Challenges/Issues/Concerns

- Established role of TRPV4 ion channels as targets. Activation of TRPV4 in skin keratinocytes can attenuate barrier dysfunction that is caused by mechanical stress. Will overcome recruitment challenge to enroll military veterans.

Budget Expenditure to Date

Projected Expenditure: \$374,984.99

Actual Expenditure: \$374,984.99 (\$245,209.39 direct cost)

Updated: October 29, 2015